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p38 Mitogen-activated Protein Kinase Regulates Oscillation of Chick Pineal Circadian Clock*

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Extracellular signal-regulated kinase (ERK) and p38 are members of the mitogen-activated protein kinase (MAPK) family, and in some cases these kinases serve for closely related cellular functions within a cell. In a wide range of animal clock structures, ERK plays an important role in the circadian time-keeping mechanism. Here we found that immunoreactivity to p38 protein was uniformly distributed among cells in the chick pineal gland. On the other hand, a constant level of activated p38 was detected over the day, predominantly in the follicular and parafollicular pinealocytes that are potential circadian clock-containing cells. Chronic application of SB203580, a selective and reversible inhibitor of p38, to the cultured chick pineal cells markedly lengthened the period of the circadian rhythm of the melatonin release (up to 28.7 h). Noticeably, despite no significant temporal change of activated p38 level, a 4-h pulse treatment with SB203580 delayed the phase of the rhythm only when delivered during the subjective day. These results indicate a time-of-day-specific role of continuously activated p38 in the period length regulation of the chick pineal clock and suggest temporally separated regulation of the clock by two MAPKs, nighttime-activated ERK and daytime-working p38.

In a variety of organisms, daily rhythms in physiology and behavior are controlled by circadian clocks, which oscillate with a period of ~24 h under constant conditions. These clocks are entrained to environmental time cues such as light and temperature (1). Genetic and molecular analyses revealed that the circadian oscillators in *Drosophila* and mice share similar positive and negative regulators forming transcription/translation-based feedback loops (2). In *Drosophila*, a complex of basic helix-loop-helix-PAS (Period-Arnt-Single-minded) transcription factors, dCLOCK and CYCLE (dBMAL1), positively regulates transcription of both period (*per*) and timeless (*tim*) genes (3–5). The complex of PER and TIM inhibits dCLOCK:CYCLE-mediated transcription (4), and thus they act as negative elements of the feedback loop in which the mRNA and protein levels of these negative elements oscillate in a circadian manner. It is now postulated that the molecular framework of the circadian oscillator is conserved from flies to mammals, although some of the clock components appear to play slightly different roles (2).

In addition to the transcriptional and translational regulation, posttranslational events such as protein phosphorylation regulate the circadian clock functions. In *Drosophila*, DOUBLETIME, a homologue of mammalian casein kinase Iε (CKIε),[1] reduces the stability of PER by phosphorylation (6, 7) and glycogen synthase kinase-3/shaggy phosphorylates TIM and regulates nuclear translocation of PER:TIM complex (8). Missense alleles of double-time or shaggy gene result in alteration of the period length of the circadian rhythm (6–8), demonstrating important roles of the protein kinases in determination of the period length. Evidence for a similar role of CKI in the mammalian clock system is also presented. That is, CKIε (and CKIδ) regulates subcellular localization and stability of PER proteins by phosphorylation (9–13), and defect in CKIε leads to the short period phenotype of *Tau* mutant hamster (14). In addition to PER proteins, the positive regulators such as BMAL1 and CLOCK also seem to be phosphorylated *in vivo* (9). Recent biochemical studies showed that CKI and extracellular signal-regulated kinase (ERK), a member of mitogen-activated protein kinase (MAPK) family, phosphorylate BMAL1 and regulate BMAL1:CLOCK-mediated transactivation (15, 16). Interestingly, ERK activity exhibits circadian rhythm in various clock-containing structures (17–20), and transient inhibition of the nighttime-activation of ERK induces a phase shift of the clock in the chicken pineal gland and bullfrog retina (18, 19), suggesting important contribution of ERK to the clockworks.

p38 is another member of the MAPK family and was originally identified as stress-activated protein kinase (21). It should be noted that the circadian rhythm of the chick pineal cells is perturbed by various cellular stresses such as osmotic stress and temperature shifts (22–25). Therefore, we directed our attention to p38 and found that a constant amount of activated p38 was present in the follicular and parafollicular pinealocytes throughout a day. We further investigated the role

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[1] The abbreviations used are: CKIε, casein kinase Iε; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; LD, 12-h light/12-h dark; CT, circadian time; CREB, cAMP response element-binding protein; ATF, activating transcription factor; CHOP, C/E-box-binding protein homologous protein; MAPKAPK, MAPK-activated protein kinase.

of p38 in the cultured chicken pineal cells using SB203580, a specific and reversible inhibitor of p38 (26, 27). We found that SB203580 treatment dramatically lengthens the free-running period of the melatonin secretion rhythm. Importantly, the phase-delaying effect of SB203580 was observed only during the subjective day, and this sharply contrasts to the nighttime-specific phase-delaying effect of PD98059, an inhibitor for ERK activation (18). We concluded that p38 is the daytime-working accelerator of the chick pineal clock.

EXPERIMENTAL PROCEDURES

Animals—Animals were treated in accordance with the guidelines of The University of Tokyo. Newly hatched male chicks were purchased from local suppliers and raised under 12-h light/12-h dark (LD) lighting conditions with the light intensity of 300 lux at the level of the head of the chick.

Immunoblot Analyses of the Pineal Gland—Pineal glands isolated from eight chicks were lysed in 500 μl of SDS-polyacrylamide gel electrophoresis sample buffer. This procedure for the dark-period sample was performed under dim red light (>640 nm). The sample was subjected to immunoblotting with anti-phospho-p38 antibody (1:1000 dilution, New England Biolabs) that detects the dually phosphorylated form of p38. The protein levels of p38 were evaluated by immunoblotting with anti-p38 antibody (1:5000 dilution, Santa Cruz Biotechnology), and the immunoreactivities were developed by CDP-Star detection system (New England Biolabs) with alkaline phosphatase-conjugated secondary antibody (1:5000 dilution, New England Biolabs). The immunopositive band density was quantitated by densitometric analysis using MacBAS 2.5 software (Fuji film).

Immunohistochemistry—The chick pineal glands were isolated under dim red light (>640 nm) and fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (10 mM Na-phosphate, 140 mM NaCl, 1 mM $MgCl_2$, pH 7.4) containing 1 mM NaF, and 1 mM Na_3VO_4 for 1 h at 4 °C. Then the tissues were cryoprotected with 20% sucrose in phosphate-buffered saline and embedded in the OCT-mounting medium (Sakura). The frozen sections (10-μm thickness) were incubated with a blocking solution (5% (w/v) bovine serum albumin, 0.2% (v/v) Triton X-100, 25 mM Tris-HCl, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM NaF, and 1 mM Na_3VO_4, pH 7.4) for 1 h at room temperature and then incubated for 48 h at 4 °C with a primary antibody diluted in the blocking solution followed by incubation for 48 h at 4 °C with Alexa 488-conjugated goat anti-rabbit IgG (1:2000 dilution, Molecular Probes) diluted in the blocking solution. The primary antibodies used were anti-phospho-p38 antibody (1:30 dilution, New England Biolabs) or anti-p38 antibody (1:300, Santa Cruz Biotechnology). TO-PRO-3 (0.2 μM, Molecular Probes) was used for staining of cell nuclei. The sections were viewed by a confocal laser-scanning microscope (TCS-NT, Leica).

Quantification of Melatonin Released from Cultured Chick Pineal Cells—The pineal glands isolated from 1-day-old chicks were mechanically disrupted by passing through a nylon mesh (pore size 100 μm), and the cells were dispersed in Medium 199 (Invitrogen) supplemented with 10 mM HEPES, 100 units/ml penicillin G (Meiji), 100 μg/ml streptomycin (Meiji), 3 μM cytosine β-D-arabinofuranoside (Kojin), 10% fetal bovine serum (JRH Biosciences), and 2.2 mg/ml $NaHCO_3$. Pineal cells were plated onto 24-well plates (Cloning Plate number 704160, Greiner) at a density of 4×10^5 cells/well and cultured at 39.5 °C under 5% CO_2, 95% air in an incubator equipped with a fluorescent lamp (300 lux at dish level). The cultured cells were kept in LD cycles from the day of plating (day 1), and the culture medium was exchanged at zeitgeber time 11 (zeitgeber time 0 is lights on, and zeitgeber time 12 is lights off) on days 3 and 5. On day 6, the culture was transferred to constant darkness, and the culture medium was exchanged at 4-h intervals under infrared light (>800 nm) with the aid of dark-field goggles (NEC) for determination of the secreted melatonin concentration in the medium. Melatonin in the culture medium was separated and quantified by using NANOSPACE SI-1 high pressure liquid chromatography system (Shiseido) as described previously (18). The phase of the rhythm of secreted melatonin levels was represented by the phase reference point (28, 29), which was defined for each peak as the time midway between the half-rise and half-fall of the peak. The half-rise point was calculated by interpolating the two time points that gave (i) the lowest melatonin concentration preceding the peak of the cycle and (ii) the peak melatonin concentration of the cycle. The half-fall point was calculated by interpolating the two time points that gave (i) the peak melatonin concentration and (ii) the lowest melatonin concentration following the peak of the cycle. The period length of a cycle was defined as the time between two consecutive phase reference points. For every individual culture, two consecutive period lengths were calculated form the first three cycles and they were averaged to determine the period length for that culture. The period lengths thus determined for three replicate cultures of one treatment group were averaged to determine the mean period length of the treatment group. The phase angles of the three cultures within a treatment group were averaged for the second and third cycle following the drug treatment. The mean phase angle of the treatment group was subtracted from that of the control group to determine the magnitude of the phase shift.

Preparation of Lysate of Cultured Chick Pineal Cells—Pineal cells were plated onto 24-well plates (BD Biosciences) at a density of 4.4×10^5 cells/well and cultured as described above. The cells were treated with either SB203580 (in 0.1% Me_2SO) or 0.1% Me_2SO (vehicle) from CT2 or CT14 on day 6 (see "Results and Discussion"). After 4-h treatment, cells were lysed immediately with 120 μl of SDS-polyacrylamide gel electrophoresis sample buffer, and the lysate was then subjected to immunoblotting with anti-phospho-CREB antibody (1:1000 dilution, New England Biolabs), anti-phospho-ATF-2 antibody (1:500 dilution, New England Biolabs), anti-phospho-MAPKAPK2 antibody (1:250 dilution, New England Biolabs), or anti-MAPKAPK2 antibody (1:500 dilution, New England Biolabs). The immunoreactive signals were developed by Renaissance chemiluminescence reagent (PerkinElmer Life Sciences) with peroxidase-conjugated secondary antibody (1:5,000 dilution, Kirkegaard and Perry Laboratories).

RESULTS AND DISCUSSION

Activity of p38 in the Chick Pineal Gland—Various cellular stresses are known to perturb the circadian rhythm of melatonin release from the chick pineal cells in culture (22–25). The phase-response curves to high temperature treatment and osmotic stress resemble that to the photic stimulation (22, 24, 25), raising the possibility that the photic entrainment of the pineal clock may share signaling molecule(s) with the stress signaling pathway. We first evaluated a light-dependent change of the chick pineal p38 activity by using the anti-phospho-p38 antibody specific to the active form of p38 (that is phosphorylated on both regulatory threonine and tyrosine residues). Chicks entrained to LD cycles were transferred to constant darkness, and the pineal glands were isolated before and after the light exposure (300 lux) of the animals at CT6, CT14, or CT18 (CT0 and CT12 refer to the times of lights on and off in the previous LD cycle, respectively). At CT6, light exposure caused ~2-fold increase of active p38 level within 10 min, which then returned to the basal level within 30 min (Fig. 1*A*, *top* and *bottom panels*). The protein levels of chick pineal p38 were almost constant before and after the light exposure (Fig. 1*A*, *middle panel*). The light stimulation at CT14 (Fig. 1*B*) or CT18 (Fig. 1*C*) had an effect similar to that observed at CT6 (Fig. 1*A*), demonstrating that p38 was phosphorylated and activated transiently after receiving light at any time of the day tested.

In the light-stimulation experiment, we noticed that a certain level of active p38 was present in the pineal gland of the chicks kept in the dark (prior to light stimulation) at CT6, CT14, and CT18 (Fig. 1). This result led us to examine circadian variation in levels of active p38 in constant dark condition. Chicks entrained to LD cycles were transferred to constant darkness, and the pineal glands were isolated at 4-h intervals over a day. As shown in Fig. 2*A*, a constant amount of active p38 was detected in the pineal homogenate prepared at every time point, and the total amount of p38 protein showed no detectable change during the period. To examine the cellular distribution of active p38 and its possible temporal change, thin sections of the pineal gland were prepared from the chicks maintained in constant darkness and they were subjected to immunostaining with the antibodies to p38 protein and to its phosphorylated form. Between the two time points tested (CT6 and CT18), no significant difference was observed in distribution of either p38 protein (Fig. 2*B*, *top row*) or its active form

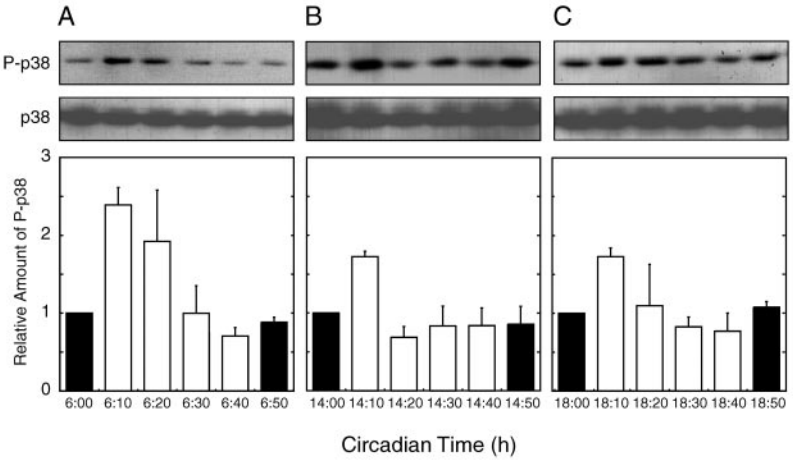

FIG. 1. **Light-activation of p38 in the pineal gland.** Chicks entrained to LD cycles for 14 days were transferred to constant darkness and exposed to white light (300 lux) at mid-subjective day (CT6, *A*), early subjective night (CT14, *B*), or mid subjective night (CT18, *C*) on day 15. Their pineal glands were isolated 10, 20, 30, or 40 min after the light onset. The pineal glands of control animals kept in the darkness were isolated at CT6:00, 6:50, 14:00, 14:50, 18:00, and 18:50. The chick pineal homogenate (20 μg) prepared at each time point was immunoblotted by anti-phospho-p38 antibody (*top panels*) and then reprobed with anti-p38 antibody (*middle panels*). The *bottom panels* indicate quantitated band densities of phosphorylated p38 in the pineal glands of animals kept in the darkness (*black bars*) or exposed to light (*white bars*). The values (mean ± S.E., n = 2–3) are relative to the band density before the light onset.

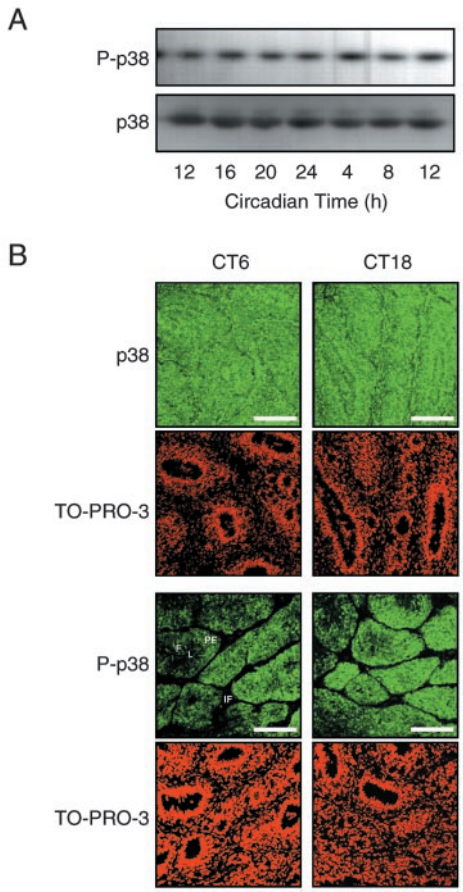

FIG. 2. **Temporal profiles of the relative amount and localization of active form of p38 in the chick pineal gland.** Chicks entrained to LD cycles for 14 days were transferred to constant darkness. *A*, the pineal glands were isolated every 4 h from days 15 to 16, and the pineal homogenate (20 μg) prepared at each time point was immunoblotted with anti-phospho-p38 antibody (*upper panel*) and then reprobed with anti-p38 antibody (*lower panel*). *B*, thin frozen sections (10-μm thickness) were prepared from the chick pineal gland fixed at CT6 or 18 on day 15 and immunostained with anti-p38 MAPK antibody or anti-phospho-p38 MAPK antibody. TO-PRO-3 (Molecular Probes) was used for staining of cell nuclei. *L*, lumen; *F*, follicular layer; *PF*, parafollicular layer; *IF*, inter-follicular cells. Scale bars, 50 μm.

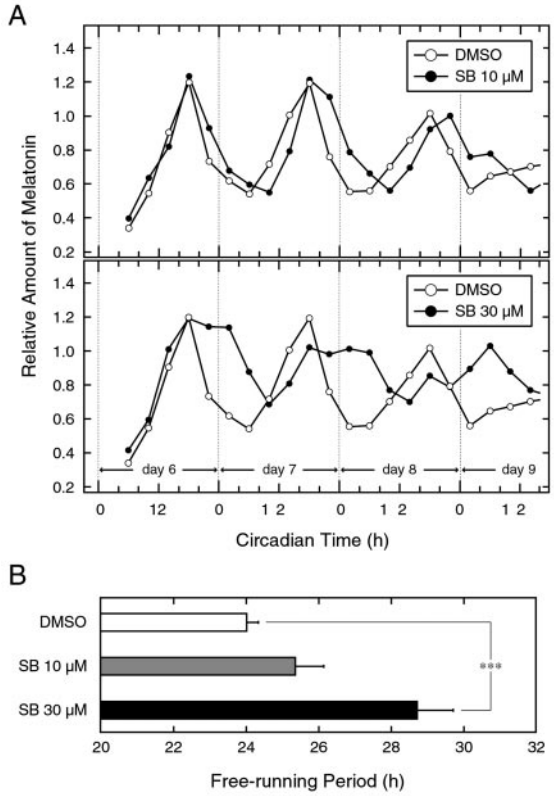

FIG. 3. **Effect of chronic application of SB203580 on the melatonin rhythm of the cultured pineal cells.** Dispersed chick pineal cells were cultured in LD cycles for 5 days and then transferred to constant darkness. *A*, the cells were treated with SB203580 (10 μM in *upper panel* or 30 μM in *lower panel*) in 0.1% Me_2SO (*DMSO*) from CT2 on day 6. Control cells were exposed to 0.1% Me_2SO from the same time (*open circles*). The melatonin level at each time point was plotted after normalization by the average value of melatonin released during the entire experimental period. The data are representative results of three independent cultures. *B*, the mean period lengths were calculated from the melatonin rhythms (see "Experimental Procedures") and are shown as mean ± S.E. of three replicate culture wells. Significance was determined using Student's t test (***, $p < 0.0001$).

(*third row*). Interestingly, p38 protein was detected in almost all of the pineal cells, whereas the active form of p38 was detected in cells within the follicles but not in the interfollicular

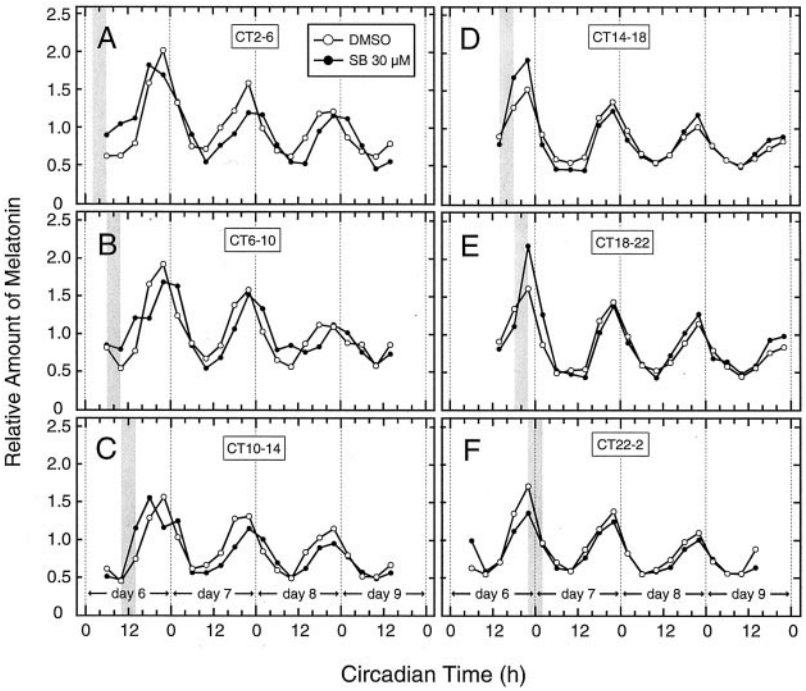

FIG. 4. **Phase-dependent effect of a pulse application of SB203580 on the melatonin rhythm of the cultured pineal cells.** The pineal cells cultured in LD cycles for 5 days were transferred to constant darkness and then treated with 30 μM SB203580 in 0.1% Me_2SO (*DMSO*) (*solid circles*) or with 0.1% Me_2SO (*open circles*) for 4 h at six different time periods (shown in the *panels A–F*) during days 6–7. The period of the 4-h treatment is indicated by the *gray zone* in each panel, and the melatonin levels are plotted as in Fig. 3. The data are representative results of three independent cultures, and Fig. 5 summarizes the data from all of the cultures.

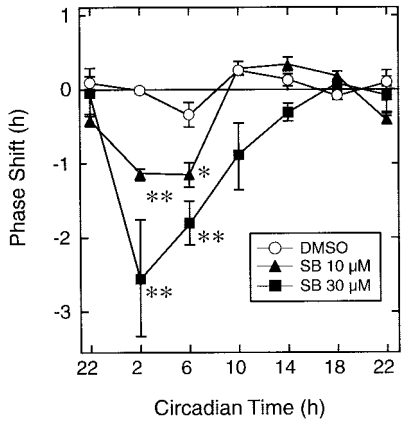

FIG. 5. **Phase-response curves for the pulse application of SB203580.** The phase shift was calculated from the difference in phase angle of the melatonin rhythm between the untreated and treated cell culture, which was exposed to 0.1% Me_2SO (*open circles*) and 10 or 30 μM SB203580 in 0.1% Me_2SO (*solid triangles* or *squares*, respectively) for 4 h from the indicated time point. Each value is mean ± S.E. of three replicate culture wells. The magnitudes of the phase advance or delay are shown as positive or negative values, respectively. Significance of the difference in magnitude of the phase shift between SB203580-treated cells and Me_2SO-treated cells at each time point was determined by using two-way analysis of variance followed by Bonferroni's *post hoc* test. (*, $p < 0.01$; **, $p < 0.001$).

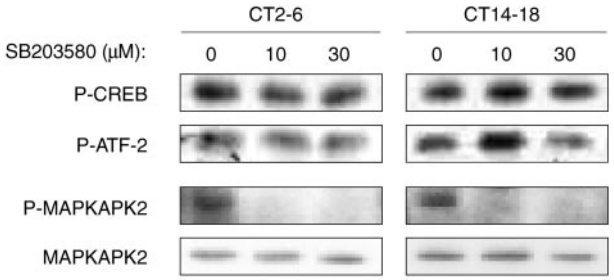

FIG. 6. **Effect of SB203580 on phosphorylation levels of candidates for p38 target.** Dispersed chick pineal cells were cultured in LD cycles for 5 days and then transferred to constant darkness. The cells were treated with 0.1% Me_2SO (*DMSO*) (0 μM) and 10 or 30 μM SB203580 (in the presence of 0.1% Me_2SO) on day 6 during CT2–6 or during CT14–18. The lysate was prepared after the 4-h drug treatment and immunoblotted with anti-phospho-CREB antibody (*top row panels*), anti-ATF-2 antibody (*second row panels*), anti-phospho-MAPKAPK2 antibody (*third row panels*), or anti-MAPKAPK2 antibody (*bottom panels*). The data are a representative set of results from two independent experiments with three sets of independent cultures.

cells (designated *IF* in Fig. 2*B*). Each follicle was composed of two types of active p38-immunopositive cells forming concentric cell layers. In the inner layer (follicular layer; Fig. 2*B*, *F*), follicular pinealocytes protrude their rudimentary outer segments into the lumen (Fig. 2*B*, *L*), and parafollicular pinealocytes in the outer layer (parafollicular layer; Fig. 2*B*, *PF*) surround the follicular pinealocytes. It was previously shown that both of the pinealocytes have photoreceptor pinopsin (30, 31) and enzymes in the melatonin biosynthetic pathway such as *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase, which are known to colocalize with the circadian pacemaker (32–34). Taken together, it was suggested that a constant amount of p38 is kept activated in the pineal clock-containing photosensitive cells.

Effect of SB203580 on the Circadian Rhythms of Melatonin Release—To explore a possible contribution of continuously activated p38 to the pineal clockwork, we examined how chronic inhibition of p38 activity alters the free-running rhythm of the melatonin release. For this purpose, we used SB203580, a specific and reversible inhibitor of p38 (26, 27). The cultured pineal cells were treated with SB203580 continuously from CT2 in constant darkness. Application of 10 μM SB203580 induced a free-running rhythm with a prolonged period of 25.2 ± 0.7 h, and application of 30 μM SB203580 remarkably deformed the rhythm with an apparent period length of 28.7 ± 1.1 h (Fig. 3, *A* and *B*). On the other hand, the free-running period of Me_2SO-treated cells and that of non-treated cells were both close to 24 h (24.3 ± 0.8 h and 24.0 ± 0.3 h, respectively, Fig. 3*B*). We concluded that p38 plays a crucial role in maintenance of the period length of the pineal clock.

Our observations together suggest that p38 may have a continuous phase-advancing effect on the clock over the day. To test this idea, we examined the effect of 4-h pulse treatment

with SB203580 on the clock. The cultured cells treated with 30 μM SB203580 during the early subjective day (CT2–6) exhibited a rhythm delayed by 2.6 ± 0.8 h (Figs. 4*A* and 5) as compared with non-treated control cells, and the drug treatment at the late subjective day (CT610) induced a smaller phase delay by 1.8 ± 0.3 h (Figs. 4*B* and 5). Noticeably, the drug treatment during the subjective night (CT14–18 and CT18–22) had a minimal effect, if any, on the melatonin rhythm (Figs. 4, *D* and *E*, and 5). The phase-dependent phase-delaying effect of SB203580 was also dependent on the concentration of the drug (Fig. 5). This all-delay phase-response curve is consistent with the period-lengthening effect of chronic application of the drug (Fig. 3) and indicates a daytime-specific function of p38 in the clock regulation.

SB203580-dependent Inhibition of Phosphorylation of p38 Substrate in the Chicken Pineal Cells—To verify the p38 activity in the daytime (and also in the nighttime), we investigated SB203580-dependent change in phosphorylation states of p38 substrates. p38 is known to phosphorylate and thereby activate various transcription factors and protein kinases such as CREB, ATF-2, myocyte enhancer factor 2C, CHOP, and MAPKAPKs (35), which could be candidates to transmit the p38 signal to the core feedback loop in the chicken pineal circadian oscillator. By using active (phosphorylated) form-specific antibodies, we examined the phosphorylation states of CREB, ATF-2, and MAPKAPK2 in the lysate of the cultured pineal cells that had been treated with 10 or 30 μM SB203580 at CT2–6 (daytime treatment) or CT14–18 (nighttime treatment) (Fig. 6). In control cultures (vehicle treated with 0.1% Me_2SO), certain amounts of the three proteins were found to be in the phosphorylated state at CT6 and CT18 (Fig. 6, *upper three row panels*, *lane 0*) and no significant difference was observed in phosphorylation level of each protein between the two time points. In both phases, the 4-h treatment with 10 or 30 μM SB203580 had no measurable effect on the phosphorylation states of pineal CREB (*top row panels*) and ATF-2 (*second row panels*). On the other hand, the phosphorylated form of MAPKAPK2 became almost undetectable after 4-h treatment at both time periods with the drug not only at 30 μM but also at 10 μM (*third row panels*) with its protein level unaltered upon the drug treatment (*bottom panels*). These results strongly suggest pineal MAPKAPK2 as a downstream target of p38 and support the presence of continuously activated p38 in the cultured cells, similar to *in vivo* situation (Fig. 2*A*). However, the results obtained do not account for the phase-dependent effect of SB203580. Also, apparently complete inhibition of MAPKAPK2 phosphorylation caused by the lower concentration (10 μM) of SB203580 (Fig. 6) is at odds with the dose-dependent effect of SB203580 on the period length (Fig. 5). These issues appear to argue against a role of MAPKAPK2 in the clock regulation, but we should note the difference in experimental design between the rhythm measurement (Figs. 4 and 5) and the phosphorylation state assay (Fig. 6). The latter is the evaluation of the final state of MAPKAPK2 phosphorylation after 4-h drug treatment, whereas the former reflects the sum of accumulative effect of the drug on phosphorylation inhibition processes that would proceed progressively during 4-h treatment. Because it is difficult to compare accurately ongoing phosphorylation inhibition processes under various conditions (daytime *versus* nighttime treatment, 10 *versus* 30 μM drug), it remains to be elucidated whether or not MAPKAPK2 may play an important role in the clock period regulation.

A Role of p38 in the Pineal Clockwork—In this study, we demonstrated that chronic treatment of the cultured chick pineal cells with p38 inhibitor (SB203580) dramatically lengthened the period length of the circadian rhythm of melatonin release (Fig. 3). Despite the presence of continuously activated p38 in the follicular and parafollicular cells (Fig. 2), the phase-delaying effect of SB203580 was observed only when applied during the subjective day (Figs. 4 and 5). We speculate that the time-of-day-specific effect of SB203580 could be attributed to a temporal fluctuation (in amount and/or distribution) of the downstream target of p38. As described above, SB203580 treatment significantly reduced the amount of phosphorylated MAPKAPK2. However, similar amounts of phosphorylated MAPKAPK2 were detected at CT6 and CT18, and SB203580 treatment decreased the phosphorylation level in a phase-independent manner (Fig. 6, *third row panels*). Given that MAPKAPK2 contributes to the clock regulation, the amount or distribution of a substrate downstream of MAPKAPK2 would change in a circadian manner. Alternatively, p38 might phosphorylate and regulate other molecule(s) such as myocyte enhancer factor 2C, CHOP, or a component of the feedback loop of the oscillator. In clock-containing cells, activities and/or protein levels of several clock gene products exhibit prominent circadian rhythms (2). In the chick pineal gland, the mRNA level of *Per2* gene peaks at CT2 and decreases during the subjective day (36). Because the transcription of *Per2* gene is positively regulated by BMAL1:CLOCK heterodimer (36), the transactivation by BMAL1:CLOCK heterodimer should be declining or suppressed in the late subjective day in the pineal gland. Therefore, it is possible that p38 or MAPKAPK2 accelerates the decline of BMAL1:CLOCK transactivation by phosphorylating the complex of these positive regulators and thereby advances the clock phase. Likewise, p38 or MAPKAPK2 may phosphorylate the negative elements, PER and CRY (37), to enhance their ability to inhibit the transactivation of BMAL1:CLOCK heterodimer. Downstream molecules of p38 or MAPKAPK2 in the pineal gland are to be investigated for understanding how p38 regulates the circadian oscillatory mechanism in the clock system.

We previously demonstrated that the inhibition of ERK activity delayed the phase of the clock during the subjective night (18), a period when SB203580 application had almost no effect on the pineal clock (Figs. 4 and 5). In addition, ERK is activated during the nighttime and it is rapidly dephosphorylated by light stimulation in the night (18). This contrasts with light-induced activation of p38 (Fig. 1). Such a contrasting control of ERK and p38 signaling pathways may be important for the proper clockwork in the chick pineal gland. Considering the strong evolutionary conservation of the MAPK family in vertebrates (38), it will be of great interest to compare the molecular frameworks of the signaling mediated by ERK and p38 in other vertebrate clock systems.

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